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Differential binding between volatile ligands and major urinary proteins due to genetic variation in mice

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ABSTRACT

Two different structural classes of chemical signals in mouse urine, i.e., volatile organic compounds (VOCs) and the major urinary proteins (MUPs), interact closely because MUPs sequester VOCs. Although qualitative and/or quantitative differences in each chemical class have been reported, previous studies have examined only one of the classes at a time. No study has analyzed these two sets simultaneously, and consequently binding interactions between volatile ligands and proteins in urines of different strains have not been compared. Here, we compared the release of VOCs in male urines of three different inbred strains (C57BL/6J, BALB/b and AKR) before and after denaturation of urinary proteins, mainly MUPs. Both MUP and VOC profiles were distinctive in the intact urine of each strain. Upon denaturation, each of the VOC profiles changed due to the release of ligands previously bound to MUPs. The results indicate that large amounts of numerous ligands are bound to MUPs and that these ligands represent a variety of different structural classes of VOCs. Furthermore, the degree of release in each ligand was different in each strain, indicating that different ligands are differentially bound to proteins in the urines of different strains. Therefore, these data suggest that binding interactions in ligands and MUPs differ between strains, adding yet another layer of complexity to chemical communication in mice.

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1. Introduction

Mice secrete a variety of chemical substances into their urine that serve as signals in social communication. Two different structural classes of chemical signals have been investigated for several decades: i.e., volatile (odorous) compounds and the major urinary proteins (MUPs). Among the huge variety of volatile organic compounds (VOCs) present in urine, some have been identified as pheromones that are involved in social and sexual communications (reviewed in [1] and [2]), whereas others have been implicated in other social contexts such as individual recognition (see [3] and references therein). In addition, exceptionally large amounts of proteins are present in male mouse urine. In particular, one class of proteins, the MUPs, account for 99% of urinary proteins [4]. Variation in the structure of MUPs is extensive in wild populations where they have been reported to be involved in individual recognition, inbreeding avoidance, and evaluation of genetic heterozygosity of potential mates (see [5] and references therein).

Although the chemical properties of VOCs and MUPs are different, they appear to interact closely since MUPs, a member of the lipocalin family, have a hydrophobic pocket that sequesters some volatile ligands [6]. Notably, most of the reported male-derived volatile pheromone molecules (e.g., 2-*sec*-butyl-4,5-dihydrothiazole [SBT], 3,4-dehydro-*exo*-brevicomin [DHB], α- and β-farnesene, and 6-hydroxy-6-methyl-3-heptanone [HMH]) have been identified as ligands of MUPs [7–9]. It has been suggested that the binding of these signaling molecules to MUPs delays their release and helps provide stable, long-lasting signals [10,11].

Previously, qualitative and/or quantitative differences in VOCs and MUPs have been observed in the urines collected from male mice of different laboratory inbred strains. Each member of an inbred strain is genetically identical, whereas there are large genetic (and phenotypic) differences among strains depending in part on the founder origin and selective pressures maintaining the strain [12,13]. For example, the levels of SBT were reported to be higher in C57BL/6 (B6) mice than in AKR, ICR or Kunming mice [14,15], whereas SBT was not detected in

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the DBA/2Crl strain [16]. Differences in the levels of DHB have also been reported among different strains [14,15,17]. Distinctive MUP profiles with qualitative and quantitative differences have been observed among laboratory inbred strains as well [18–21]. Notably, an atypical MUP isoform, known as darcin, that preferentially binds SBT, is exclusively expressed in the male urines of the inbred laboratory mice derived from the C57 lineage (e.g., B6), but not in most of those derived from the Castle lineage (e.g., BALB/c and AKR) [20,22,23]. In addition, quantitative differences of VOCs and MUPs were observed in the male urines derived from two different subspecies of the house mice (*Mus musculus musculus* and *Mus musculus domesticus*) [24,25].

Previous studies of strain (and subspecies) differences in the urine-based chemical signals have examined only one of the chemical classes at a time. To date, no study has analyzed MUPs and VOC ligands simultaneously. Consequently, binding interactions between ligands and proteins in urines of different strains have not been compared. We hypothesized that there may be an added level of complexity to chemical signals that distinguish inbred mouse strains (and hence, individual outbred mice in nature) due to differential binding of ligands and MUPs. To test this hypothesis, we compared the release of VOCs in male urines of three different inbred strains (C57BL/6J, BALB/b and AKR) before and after denaturation of urinary proteins. We also examined the profile of MUPs in each strain as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing gel electrophoresis.

2. Materials and methods

2.1. Mice

Adult males of three inbred mouse strains were used in this study. Eight C57BL/6J (B6), 7 AKR and 6 BALB/b mice were born and raised in our mouse colony and used as urine donors when they were between 3 and 10 months of age. The mice were kept in a temperature-controlled room at 23 °C on a 12:12-h light–dark cycle. Two to three mice of the same strain lived together in polypropylene cages (10 cm × 28.5 cm × 12.5 cm). All mice were fed the laboratory rodent diet 5001 as purchased from Purina Mills (St. Louis, MO). All animals were maintained under uniform conditions and cared for in accordance with the Guide for the Care and Use of Laboratory Animals and the experimental protocols were approved by the Institutional Animal Care and Use Committee in the Monell Chemical Senses Center (Approval number: 900p).

2.2. Urine collection

Urine samples were collected repeatedly from each individual mouse by gentle abdominal pressure over 10 days and stored at −20 °C. Samples collected from the same individual were combined for the chemical analysis. Two 25 μl urine aliquots were prepared from each mouse: one was extracted intact and the other after denaturation. Most of the urine samples were analyzed within 2–3 weeks after collection of urine.

2.3. Collection of mouse urine volatiles by solid phase microextraction

Twenty five microliters of intact or denatured urine was placed in a 4 ml glass vial and a 2 cm, three-component solid phase microextraction (SPME) fiber (30 μm carboxen, 50 μm divinylbenzene, polydimethylsiloxane, Supelco Corp., Bellefonte, PA) was used for collection of the headspace VOCs in the vial. The vial was submerged in a water bath at 37 °C and was equilibrated for 10 min. Then, the headspace VOCs were extracted by the SPME fiber for 30 min at 37 °C. The urine sample in the vial was agitated using a magnetic stirrer during the entire extraction period. The SPME fiber containing the adsorbed VOCs was then inserted into the injection port of a gas chromatograph–mass spectrometer (GC–MS) and the VOCs were desorbed for 5 min at 230 °C.

2.4. Gas chromatography–mass spectrometry

A Thermo Scientific ISQ single quadrupole GC–MS (Waltham, MA) was used for separation and analysis of the desorbed VOCs. The GC–MS was equipped with a Stabilwax column (30 m × 0.32 mm with 1.0 μ film thickness; Restek, Bellefonte, PA). The following chromatographic protocol for separation before MS analyses was employed: 60 °C for 4 min, then programmed at 6 °C/min to 230 °C with a 13-min hold at this final temperature. Helium was used as the carrier gas at a constant flow rate of 1.5 ml/min. The injection port was held at 230 °C. Operating parameters for the mass spectrometer were as follows: ion source temperature at 200 °C; electron impact ionization (70 eV); and scanning frequency was 4/s from *m/z* 41 to *m/z* 300.

2.5. Denaturation of proteins in urine and monitoring ligands

To estimate the relative proportions of volatile ligands free and bound to urinary proteins, we denatured urinary proteins and compared the levels of VOCs released from urine before and after denaturation. Previously, our group reported that some VOCs whose headspace concentration increased upon denaturation were ligands released from urinary proteins [5]. The denaturation was accomplished by adding 20 mg of guanidine hydrochloride (GdmCl) into a vial containing 25 μl of intact urine. The total concentration of GdmCl in urine was 8 M. Each sample was allowed to denature for 1 h at room temperature prior to extraction of the headspace volatiles in the sample.

2.6. Data analysis

We used urine samples of 8 B6, 7 AKR and 6 BALB/b male mice. In each animal, two 25 μl urine aliquots were collected and analyzed: one was extracted intact and the other after denaturation. These analyses yielded a total of 42 total ion chromatograms (TICs). The resulting chromatograms and mass spectra were then analyzed by the Metabolite Differentiation and Discovery Lab (MeDDL [26]; http://meddl.cs.wright.edu/doku.php), a novel metabolite profiling software solution adapted for GC–MS data.

The MeDDL platform is an open source informatics package currently implemented in MATLAB v2010a (The MathWorks Inc., Natick, MA) that allows for registration of "peaks," which are defined here as a single ion or measured mass/charge (*m/z*) at a given retention time, mass and chromatographic time alignment, and a suite of statistical and pattern recognition tools selected for biomarker screening studies. In brief, the MeDDL tool reads in lists of CDF (common data format) conversions of the raw GC/MS data files, registers peaks based on user-defined parameters in terms of mass sensitivity and accuracy thresholds as well as chromatographic reproducibility tailored to the performance of the analytical platform, and performs alignment of the generated peak lists in both time and mass. Following registration and alignment, the data was analyzed using two of the principal analytical methodologies included in MeDDL: unsupervised clustering via principal component analysis (PCA [27]) and differential down selection of peaks through combination of a set of logical filters described below.

MeDDL was originally created for the analysis of liquid chromatography–mass spectrometry (LC–MS) data. The ionization techniques generally employed for LC–MS are termed "soft" and impart low energy to eluting analytes, resulting in fairly simple mass spectra: often composed of just the ionized analyte, or "parent" ion. Modifications to the original implementation of MeDDL were required to aid in the analysis of the more complex mass spectra in GC–MS resulting

from the "hard ionization" induced by the electron impact (EI) fragmentation process in the mass spectrometer's ion source. A reductionist approach for this analysis was required for the efficient determination of changes observed between sample groups. To address this issue, we created a supplementary time-binning filter allowing analysts to specify both a time window and lower bound threshold of peak intensities. The comparison then proceeded as follows: an averaged, composite image of each user-defined comparative group is generated (e.g., the surface obtained from 8 samples each comprising the B6 intact urine and the associated B6 denatured urine); the most intense peak from all comparative groups is evaluated across all aligned images using a 0.1 minute window and 100,000 absolute (total ion count) threshold; once the comparison was completed, this "time slice" based upon the peak apex ± ½ of the specified time window was removed from further analysis and the next most intense set of peaks was compared. Additional filters applied in the differential analysis of groups in this study included: a fold change filter limiting results to only those peaks which demonstrated at least 2 fold or greater change in intensity upon protein denaturation; N-way ANOVA with only those peaks having $P<0.1$ significance between intact and denatured groups; and a group intensity filter where the absolute intensity of a peak must exceed 300,000 absolute in either the intact or denatured state. Once each of these filters was applied to the grouped, global data set, a Boolean operator "AND" was added to the resulting filtered peak sets to identify the logical intersection of the peak sets, an approach similar to that used in generation of a Venn diagram. These reduced data sets were then used for further manual comparison and compound identification.

2.7. Compound identification

Compound identification was accomplished via mass spectral library comparison (NIST08) combined with manual interpretation and comparison with standard samples that were either purchased or synthesized. HMH, DHB, SBT, and 2-isopropyl-4,5-dihydrothiazole were synthesized and kindly provided by Dr Kenji Mori and their synthetic procedure is described elsewhere [28,29]. Cedrol, α- and β-cedrenes, methyl methylthiomethyl disulfide, 2-acetyl-1-pyrroline, *exo*-brevicomin, 6-methyl-3-heptanone, and 5-hepten-2-one were tentatively identified using the library as well as published literature [30–33]. Nonanal, 3-ethyl cyclopentanone, 2-*sec*-butylthiazole, 1-octen-3-ol, 4-ethylphenol, dimethyl sulfone, and α- and β-farnesenes were purchased from Sigma-Aldrich (St. Louis, MO).

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on Mini-PROTEAN TGX (Tris-Glycine eXtended) Any kD gel according to the manufacturer's instruction (BioRad, Hercules, CA). Each urine sample was diluted with phosphate-buffered saline (1:10) and Tris–Tricine sample buffer was then added to the diluted sample (1:1). Twenty microliters of the sample was heated for 5 min at 95 °C and then loaded onto the gel. A 40 mA current was applied to the gel. Protein bands were visualized with LabSafe GEL Blue stain (G-Biosciences, Maryland Heights, MO). We conducted the SDS–PAGE of 8 B6, 4 BALB/b and 4 AKR male urine samples. Each urine sample was collected from different individual mice.

2.9. Isoelectric focusing gel electrophoresis

Isoelectric focusing (IEF) gel electrophoresis was performed in XCell *SureLock* Mini-Cell (Life Technologies, Carlsbad, CA). Two microliters of urine was diluted to 10 μl with Novex® pH 3–7 IEF buffer (Life Technologies, Carlsbad, CA). The urine and buffer mixture was loaded into a Novex® precast pH 3–7 IEF gel. The gel was run at 100 V for 1 h and then at 200 V for 2 h. Protein bands were visualized with Coomassie Brilliant Blue. We conducted the IEF of 3 B6, 3 AKR and 3 BALB/b male urine samples. Each urine sample was collected from different individual mice.

3. Results

Fig. 1 shows the TICs of the headspace VOCs extracted from the intact and denatured male urine samples of different mouse strains. Different volatile profiles were observed in the intact and denatured male urine samples of the B6, BALB/b and AKR mouse strains and these differences were based on different ratios among the VOCs. In terms of the absolute intensity in the chromatograms, the major VOCs in the intact B6 urine were DHB, SBT, α- and β-farnesenes and two cyclic, dehydrated products of HMH (Fig. 1A). On the other hand, in those of AKR mice, DHB was the predominant VOC, but the levels of SBT were much smaller than those of other strains (Fig. 1). Larger amounts of 4-ethylphenol were detected in the intact BALB/b samples than in those of other strains (Fig. 1). Upon denaturation, these profiles changed. Notably, the release of SBT was evident (Figs. 1 and 2A). Since bound ligands are dissociated and released from proteins upon denaturation, this result reflects that the vast majority of SBT is bound to urinary proteins derived from the three male mouse strains. No increase of DHB was observed in the B6 urine after denaturation (Figs. 1A, B and 2B), indicating that DHB may not be bound preferentially to proteins in the B6 urine. In contrast, DHB increased in the AKR and BALB/b urines upon denaturation (Figs. 1 and 2B).

We then examined all detected peaks in the chromatograms by using MeDDL in order to monitor the changes in the release of additional VOCs. A total of 1895 peaks (ions) were registered and aligned. Note that the number of registered peaks does not correspond to an equal number of individual compounds since a single compound is generally composed of multiple peaks (ions) for the EI mass spectrum as described above in the implementation of the time binning filter. Following registration and alignment, the GC–MS data were analyzed by PCA and the above filter set (see Materials and methods for details). The PCA plots revealed some separation among six different groups as well as between intact and denatured groups (Fig. 3), indicating that both strain difference and protein denaturation are important contributors to the unique volatile profiles. Results obtained from the three strain-specific pairwise comparisons of intact to denatured samples by the intersection of the generated filter sets, displayed significant, 2 fold or greater absolute changes in 49 peaks induced by protein denaturation in AKR, 26 in B6, and 36 in BALB/b, respectively. Some of them were identified and are listed in Table 1. This table as well as Fig. 2 illustrates that the vast majority of numerous VOCs were bound to urinary proteins. These ligands represented a variety of different structural classes of VOCs, confirming the previous findings that MUPs are capable of sequestering different classes of lipophilic VOCs [34]. We then compared the degree of release in each identified ligand, i.e., the fold change of the intensity following denaturation, across strains (Kruskal–Wallis One-way ANOVA). As shown in Table 1, the degree of release in the majority of the ligands is different between strains, indicating that different ligands are differentially bound to proteins in the urines of different strains.

To determine which proteins are responsible for binding the VOCs in urine, we separated proteins in the urines of the different male mouse strains through SDS–PAGE. As shown in Fig. 4a, MUPs were the only protein bands visible in the gels in most of the samples. The absence of other proteins (e.g., albumin) in urine suggests that the proteins responsible for the binding are MUPs. The SDS–PAGE also revealed that a high mobility band corresponding to darcin [23] was present only in the B6 male urine (Fig. 4a), not in BALB/b and AKR male urine, which is consistent with the previous report [20]. While its molecular mass is 18,893 Da [23], it elutes around 16 kDa in the gels, which has been reported previously [22,23]. The IEF gel

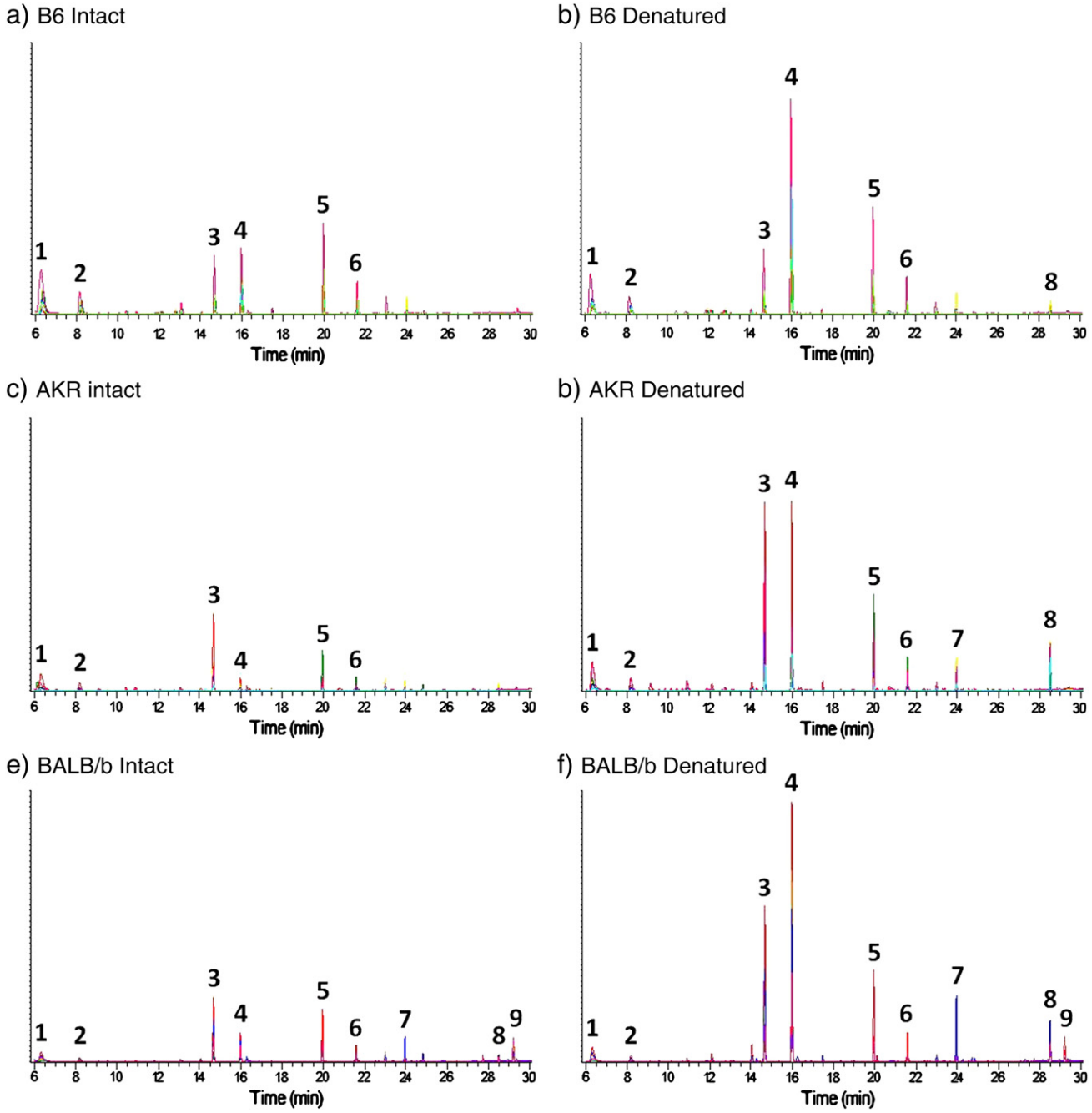

Fig. 1. The total ion chromatograms of the headspace volatile compounds extracted from the intact and denatured male urine samples of different mouse strains. The y-axis indicates absolute intensity with the same scale (for all 6 panels) and the x-axis indicates retention time in minutes. All chromatograms in each group are overlaid and designated by a different color. Prominent compound peaks include a cyclic, dehydrated product of 6-hydroxy-6-methyl-3-heptanone [HMH] (1), another cyclic, dehydrated product of HMH (2), 3,4-dehydro-*exo*-brevicomin [DHB] (3), 2-*sec*-butyl-4,5-dihydrothiazole [SBT] (4), β-farnesene (5), α-farnesene (6), Texanol (a paint-derived compound) (7), cedrol (8), and 4-ethylphenol (9).

electrophoresis revealed that B6 and AKR male urine samples had similar MUP profiles, although quantitative differences in some protein bands as measured by the band intensity were observed (Fig. 4b). The MUP profile of BALB/b determined by IEF was considerably different from those of B6 and AKR. These IEF analyses together with SDS–PAGE analyses showed polymorphisms of the major urinary proteins (MUPs) in the different inbred mouse strains. Overall, these data demonstrate that both MUP and VOC profiles are distinctive in the intact urine of each strain and further the binding interactions between volatile ligands and MUPs in urine differ between strains of inbred mice.

4. Discussion

Numerous phenotypic variations exist among laboratory inbred mouse strains. For example, differences in morphology and physiology due to variation in genes have been documented extensively; a list of inbred strains and their characteristics is available from Mouse Genome Database (MGD) maintained on the Jackson Laboratory website (http://www.informatics.jax.org). Individual urine-based olfactory signatures are not an exception. Mice can discriminate the urinary odorants of other mice with different genetic backgrounds. For example, mice have been trained to discriminate the odorants of the B6

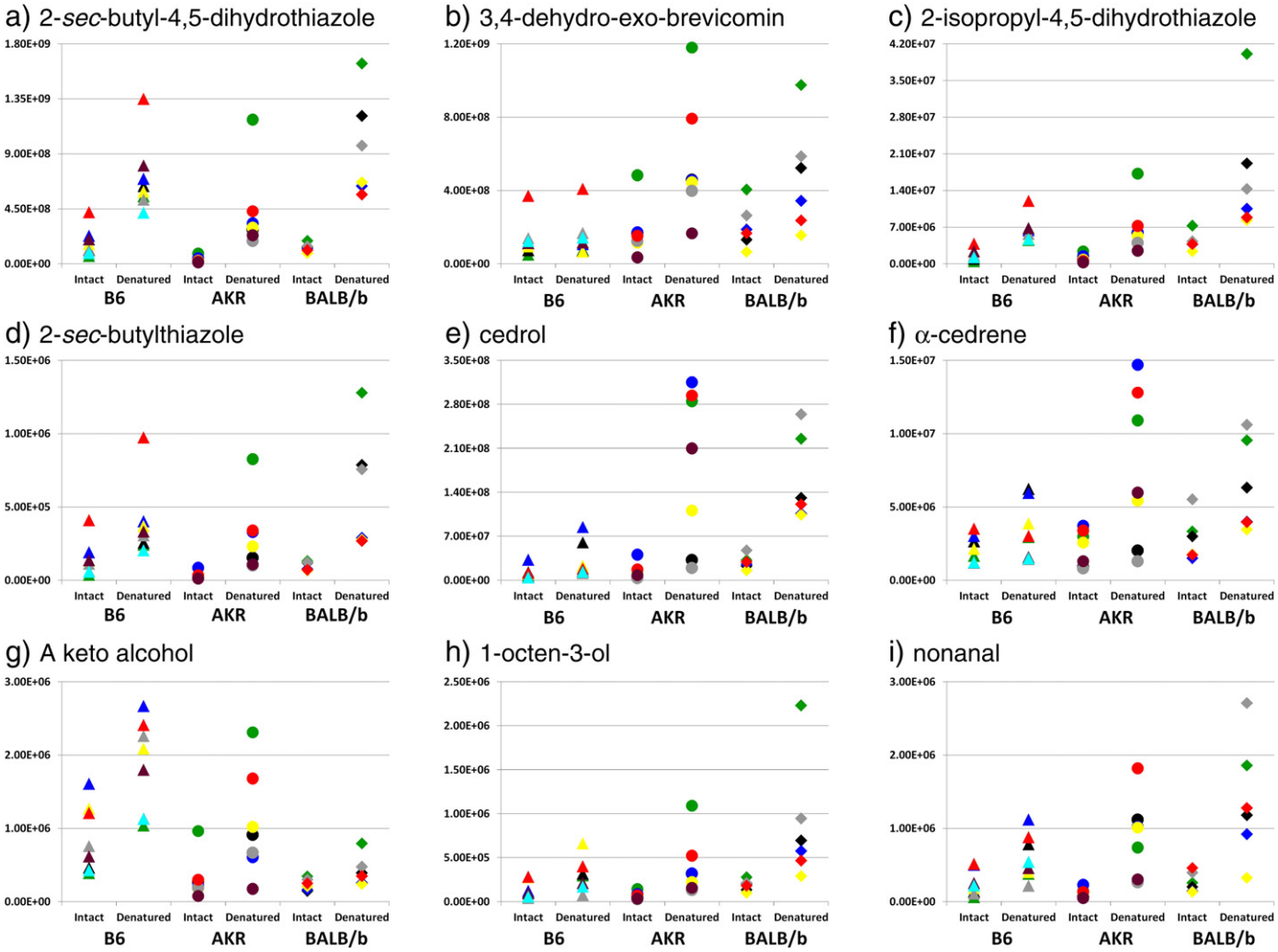

Fig. 2. Changes in the release of ligands upon protein denaturation in the urine samples derived from different male mouse strains. The y-axis indicates the absolute intensity of the total ion counts in each ligand. Each pair (intact versus denatured urine collected from the same individual mouse in each strain) is designated by the same color.

strain from those of the AKR strain as well as from many substrains of the B6 strain in our laboratory (reviewed in [35]). Pregnancy in BALB/c females mated with BALB/c males can be terminated by exposure to the urines derived from B6 males and vice versa [36]. Some of the potential components in mouse urine that underlie these odorant differences have been identified. Of these, the profiles in VOCs and MUPs differ qualitatively and/or quantitatively among inbred strains [14–20]. However, these studies examined only one set of signals (either volatiles or proteins) at a time. We are not aware of any study that has compared these two sets simultaneously.

Recently, we developed a novel method to examine volatile ligands of urinary proteins in mouse urine [5]. To identify volatile ligands associated with MUPs, previous studies employed extensive purification steps to isolate MUPs prior to extraction of ligands [7–9]. Our approach has been different. Intact urine samples are used and the changes in release of VOCs upon protein denaturation by addition of GdmCl are monitored. We previously demonstrated that some VOCs whose headspace concentrations increased upon protein denaturation were ligands released from MUPs [5]. By using this method, we compared here the release of volatile ligands in the male urines of three different inbred strains before and after denaturation of urinary proteins.

Upon denaturation, substantial increases in the release of many VOCs occurred (Figs. 1 and 2 and Table 1), which was likely due to their dissociation from the denatured proteins [5]. Our results substantiate that large amounts of VOCs are bound to urinary proteins and that these ligands represent a variety of different structural classes of organic compounds [7–9]. In addition, our data support the notion that the binding of volatile signaling molecules to MUPs slows their release from urine scent marks and thereby helps provide stable, long-lasting signals [10,11]. Furthermore, the degree of release of each ligand is different in each strain (Fig. 2 and Table 1), indicating that different ligands are differentially bound to proteins in the urines of different strains.

While numerous VOCs increased upon denaturation of urinary proteins, some compounds such as dimethyl sulfone and 2-acetyl-1-pyrroline decreased (Supplementary Fig. S1). This raises the question of whether the SPME fiber was saturated when the urine samples were extracted. To examine this question, we took different volumes (10, 50 and 100 μl) of a pooled B6 male urine sample, extracted each after denaturation by SPME, and monitored the intensity of the released SBT, the predominant ligand of urinary proteins (Fig. 1). As shown in Supplementary Fig. S2, SBT continued to increase up to 100 μl, indicating that the SPME fiber was not saturated in our extractions and analyses of 25 μl of urine, reported here. Therefore, the decrease of some VOCs was not likely due to the saturation of the SPME fiber. We think that the decrease is most likely due to the huge

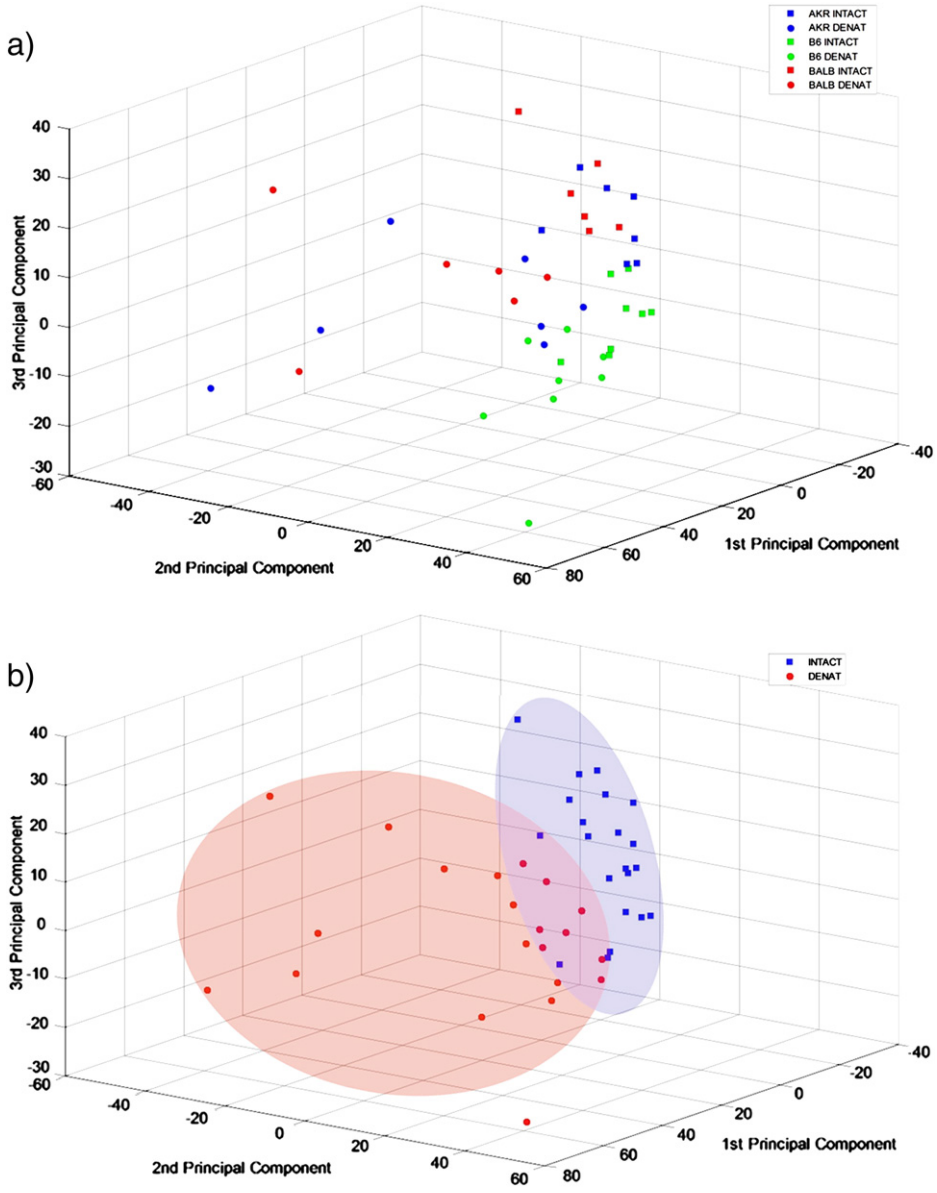

Fig. 3. Principal component analysis of the GC–MS data for the six different groups (a) and for the intact and denatured groups (b).

release of the previously bound MUP ligands (e.g., SBT and DHB). The release of these VOCs would change the partition of themselves and other VOCs between urine and the headspace, and compete for binding to the SPME fiber. Since SPME is an equilibrium extraction method, not an exhaustive extraction method, some VOCs are extracted less efficiently by the fiber after denaturation of MUPs.

The urinary proteins that bind and release VOCs are almost certainly MUPs for several reasons. First, based on SDS–PAGE analyses of the urine samples, MUPs were generally the only protein bands visible in the gels (Fig. 4a), excluding the presence of other proteins that could conceivably be responsible for the binding. Second, no study has shown that albumin (and other proteins besides MUPs) in mouse urine retains VOCs, although albumin has been reported to sequester some VOCs in the urine of female Asian elephants [37] as well as in the blood of mammals (e.g., isoflurane, a volatile anesthetic, binds to bovine serum albumin [38]). Even if albumin and other proteins were capable of capturing VOCs, their binding role in mouse urine is likely to be minor because their amounts in urine are much smaller than those of MUPs. To confirm this, we added a purified bovine serum albumin (100 μg) into 50 μl of a low molecular weight urine fraction (MW < 10 kDa) obtained by centrifugal filtration of a B6 male urine sample, and compared the release of VOCs before and after addition of albumin as well as before and after the denaturation of the fraction containing albumin. No apparent binding was observed (data not shown). Darcin, an atypical MUP isoform which preferentially retains SBT, was detected in all B6 urine samples, but absent in the BALB/b and AKR urine (Fig. 4a). The IEF together with SDS–PAGE results exhibited polymorphisms of MUPs in the different inbred mouse strains.

There are several limitations to the data we present here. First, MUPs are extremely refractory to denaturation, making it difficult to be sure that their ability to bind volatile ligands is completely eliminated. Although GdmCl is one of the most effective and widely used protein-denaturants, complete denaturation of proteins in urine may not occur upon addition of GdmCl [5]. This means that the ligands are probably not completely dissociated from urinary proteins.

Second, for the GC/MS data analyses, we used the un-normalized, absolute intensity in the base peak ion (m/z) of each compound. We explored use of the recommended quantification methods [39], but the unique chemical properties of mouse urine, namely the remarkable ability of MUPs to sequester VOCs, rendered them unreliable. For this reason, we did not compare directly the three strains for

Table 1
A list of the compounds whose absolute intensities were significantly greater after compared to before protein denaturation of the urine samples from at least one of the three inbred male mouse strains.

Compound	Avg time	Avg mass	Fold change (B6)	Fold change (AKR)	Fold change (BALB)	Strain difference[a]
A keto alcohol	7.0	55	2.37[b]	3.65[b]	1.79	0.0111
6-Methyl-3-heptanone	9.8	57	1.29	2.17[b]	1.27	0.0092
exo-Brevicomin	12.1	43	2.52[b]	13.16	7.56[b]	0.0008
An unsaturated branched ketone	12.8	57	1.62	4.37[b]	2.60	0.0006
3-Ethyl cyclopentanone	13.0	55	1.45	2.85[b]	1.57	0.0168
2-Isopropyl-4,5-dihydrothiazole	14.1	60	4.15[b]	8.04[b]	3.77[b]	0.0113
Nonanal	14.3	57	3.01[b]	8.54[b]	5.26[b]	0.0127
2-*sec*-Butylthiazole	14.4	113	3.33[b]	8.13	5.70[b]	0.0032
3,4-Dehydro-*exo*-brevicomin [DHB]	14.7	43	1.08	3.55[b]	2.38[b]	0.0004
1-Octen-3-ol	15.4	57	2.60	5.55[b]	4.33[b]	0.0133
2-*sec*-Butyl-4,5-dihydrothiazole [SBT]	16.0	115	4.72[b]	14.48[b]	7.04[b]	0.0009
A DHB-related compound	16.1	95	1.40	3.36[b]	2.44	0.0011
α-Cedrene (T)	18.3	119	1.56	3.13[b]	2.30[b]	0.0105
β-Cedrene (T)	19.0	161	2.83	14.03[b]	6.21[b]	0.0002
Methyl methylthiomethyl disulfide	20.4	61	9.88	6.82	4.43[b]	0.3581
An SBT-related compound	21.5	60	3.64[b]	3.53	4.58	0.3309
Cedrol	28.5	150	2.65	13.18[b]	5.57[b]	0.0003

[a] Comparing fold differences across inbred strains (Kruskal–Wallis One-way ANOVA).
[b] Significant ($p<0.05$, N-way ANOVA) comparing before vs. after denaturation within strain.

each VOC. Instead, we compared the release of VOCs before and after denaturation within a strain, and then compared the degree of release in each ligand, i.e., the fold change of the intensity following denaturation, across strains. We believe that these comparisons are the best way to analyze our data since two urine aliquots are analyzed simultaneously: one aliquot was extracted without GdmCl and the other with GdmCl (intact versus denatured). As shown in Table 1, the degree of release in the majority of ligands is different between strains, indicating that different ligands are differentially bound to proteins in the urines of different strains.

Third, the listed VOCs that interact with MUPs are just a fraction of ligands in mouse urine. Several ligands reported previously (e.g. farnesenes and HMH) were not included in Table 1 since their fold changes after denaturation of MUPs were less than 2 times and/or statistically insignificant. This doesn't mean that these compounds don't interact with MUPs. Furthermore, our extraction method excludes the analysis of highly volatile compounds some of which may bind to MUPs. The data acquisition begins at 6 min to avoid the interference caused by several organic solvents (e.g., chloroform, ethanol, etc.) present in our laboratory.

Fourth, the age of the animals used in this study ranged from 3 to 10 months. The age difference may influence the profiles of volatile constituents and MUPs in urine. Osada et al. [40] investigated the effect of age of B6 male mice on the urinary volatile constituents, and reported 10 compounds whose concentrations differed between adult group (3–8 M) and aged group (15–20 M). Garratt et al. [41] observed the decreased production of MUPs in the old group (25–33 M) compared to that in the younger group (12–23 M). Since these prior studies employed much older mice than our current study, however, it is not known how much the age variation influences the productions of VOCs and MUPs as well as their interactions. Future studies should examine this question.

Fifth, the male mice were caged together in groups of 2 to 3 for up to 10 months. Consequently, they may have formed social hierarchies

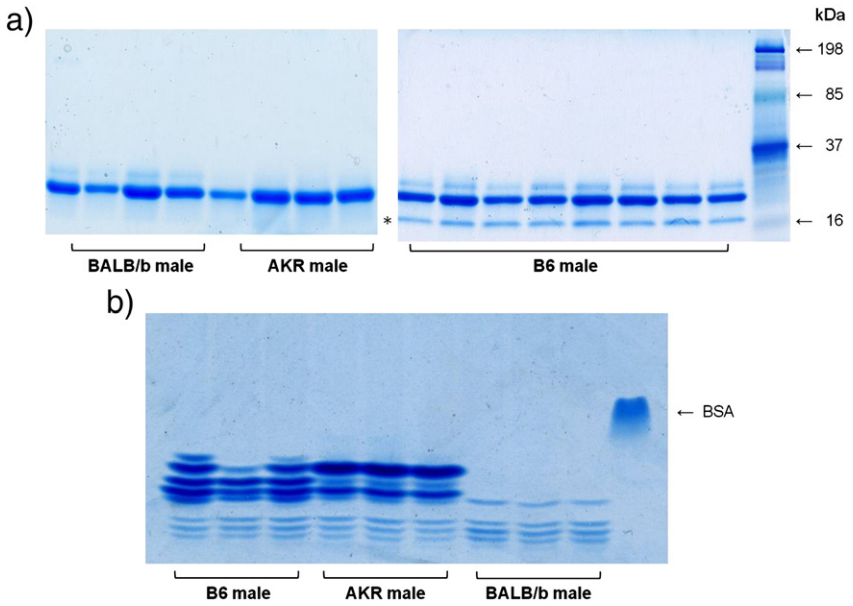

Fig. 4. Intact male urine samples resolved by SDS–PAGE (a) and IEF (b). Each lane contained a urine sample collected from different individuals. The high-mobility band in the SDS gel (*) was darcin [22] that was only present in the B6 male urine. BSA: bovine serum albumin.

which could impact on the quality and/or quantity of urinary odorants. The social status of mice (e.g., dominance) has been reported to influence volatile profiles as well as MUP profiles [42,43]. Thus, the effect if any of dominance status on the interactions between VOCs and MUPs in mouse urine remains to be determined.

Last but not least, we collected urine samples repeatedly from each mouse by gentle abdominal pressure, which might enhance the levels of the VOCs secreted from preputial glands in urine (e.g., farnesenes [44]). Nevertheless, we believe that our study demonstrates the extreme binding capacity of MUPs for volatile ligands as well as fully documents the differential binding interactions between MUPs and VOCs in different mouse strains.

What does all this mean in the context of social communication in mice? Numerous studies have been conducted to investigate the genetic bases of individual "odor signatures" (odortypes) in mice, and several candidate genes and their products have been identified. Among them, variation in major histocompatibility complex (MHC) genes as well as in genetic backgrounds influences odortypes in mice. Different urinary VOC profiles due to the variation have been observed (reviewed in [3]). Variation in the structure of MUPs in wild mouse populations has also been reported to play a significant role in individual olfactory recognition. These two distinct classes of molecules have been implicated in mouse urine-based chemical communication: small volatile molecules and proteins. Moreover, several studies have demonstrated that each class, by itself, plays a significant role. For example, some studies excluded MUPs from urine to collect VOCs only [45–47], whereas other studies employed recombinant MUPs that excluded the presence of VOCs produced endogenously by mice [23,48,49]. The interrelationship among these two classes of components in individual recognition in mice under natural circumstances remains to be elucidated.

At first glance, the amounts of odoriferous volatile ligands perceived by mice may not reflect the absolute amounts in urine since large portions of each ligand are bound to urinary proteins. Consequently, one might argue that only some fraction of the unbound (free) odorants will get to olfactory receptors. However, it is likely possible that some of the ligands bound to MUPs in urine are captured and carried by MUPs and/or other odorant-binding proteins (OBPs) present in the nose [50,51] while mice are in physical contact with urine stimuli. The same urine stimulus may be perceived differently by mice depending on whether or not the animals contact the stimulus. For example, Ramm et al. [52] reported that female mice preferred male over female urine when they contacted the urine samples, whereas no preference was observed when the mice were prohibited from contacting the stimuli. Since mice are surely exposed to both volatile and non-volatile signals while contacting urine stimuli, we suggest that the animals may perceive higher concentrations of VOCs, some of which are previously bound to MUPs, when they are in contact with urine stimuli compared with when they have no contact. But they may also be exposed to qualitatively different compounds when allowed direct contact with the urine stimulus. An alternative explanation for differential responses to urine odorants as a function of contact versus no contact has been offered by Beauchamp et al. [53]. These authors, studying guinea pigs, postulated that contact allowed molecules to reach the vomeronasal organ (VNO) and this was reinforcing, thereby inducing males' responses to female urine stimuli. When access to the VNO was prevented either by removal of the VNO [53] or by simply placing a wire screen above the urine [54], responses of males to female urine stimuli gradually declined. Sharrow et al. [50] isolated a MUP isoform present in nasal mucus as well as in the VNO of mice, and speculated that nasal MUPs may sequester volatile pheromone ligands and potentially transport them to their olfactory receptors. Therefore, it remains to be determined what proportions of volatile ligands bound to urinary MUPs are delivered into the nose when mice are in contact with urine.

Our study demonstrates the extreme binding capacity of MUPs for volatile ligands and the differential binding interactions between ligands and MUPs in different inbred mouse strains. Although the mechanism underlying these differential bindings in different strains is not known, it is most likely due to the variation in metabolism involved in the expressions of MUPs and ligands caused by genetic differences. This adds yet another level of complexity to chemical communication in mice.

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References

[1] Novotny MV. Pheromones, binding proteins and receptor responses in rodents. Biochem Soc Trans 2003;31:117–22.
[2] Tirindelli R, Dibattista M, Pifferi S, Menini A. From pheromones to behavior. Physiol Rev 2009;89:921–56.
[3] Kwak J, Willse A, Preti G, Yamazaki K, Beauchamp GK. In search of the chemical basis for MHC odourtypes. Proc Biol Sci 2010;277:2417–25.
[4] Hurst JL, Beynon RJ. Scent wars: the chemobiology of competitive signalling in mice. Bioessays 2004;26:1288–98.
[5] Kwak J, Josue J, Faranda A, Opiekun MC, Preti G, Osada K, et al. Butylated hydroxytoluene is a ligand of urinary proteins derived from female mice. Chem Senses 2011;36:443–52.
[6] Böcskei Z, Groom CR, Flower DR, Wright CE, Phillips SE, Cavaggioni A, et al. Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. Nature 1992;360:186–8.
[7] Bacchini A, Gaetani E, Cavaggioni A. Pheromone binding proteins of the mouse, *Mus musculus*. Experientia 1992;48:419–21.
[8] Lehman-Mckeeman LD, Caudill D, Rodriguez PA, Eddy C. 2-*sec*--butyl-4,5-Dihydrothiazole is a ligand for mouse urinary protein and rat alpha 2u-globulin: physiological and toxicological relevance. Toxicol Appl Pharmacol 1998;149:32–40.
[9] Novotny MV, Ma W, Wiesler D, Zídek L. Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. Proc Biol Sci 1999;266:2017–22.
[10] Hurst JL, Robertson DHL, Tolladay U, Beynon RJ. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. Anim Behav 1998;55:1289–97.
[11] Timm DE, Baker LJ, Mueller H, Zidek L, Novotny MV. Structural basis of pheromone binding to mouse major urinary protein (MUP-I). Protein Sci 2001;10:997–1004.
[12] Guénet JL. The mouse genome. Genome Res 2005;15:1729–40.
[13] Reuveni E, Birney E, Gross CT. The consequence of natural selection on genetic variation in the mouse. Genomics 2010;95:196–202.
[14] Novotny M, Jorgenson JW, Carmack M, Wilson SR, Boyes EA, Yamazaki K, et al. Chemical studies of the primer mouse pheromones. In: Müller-Schwarze D, Silverstein RM, editors. Chemical signals: vertebrates and aquatic invertebrates. New York: Plenum Press; 1980. p. 377–90.
[15] Zhang JX, Rao XP, Sun L, Zhao CH, Qin XW. Putative chemical signals about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). Chem Senses 2007;32:293–303.
[16] Röck F, Hadeler KP, Rammensee HG, Overath P. Quantitative analysis of mouse urine volatiles: in search of MHC-dependent differences. PLoS One 2007;2:e429.
[17] Willse A, Kwak J, Yamazaki K, Preti G, Wahl JH, Beauchamp GK. Individual odortypes: interaction of MHC and background genes. Immunogenetics 2006;58:967–82.
[18] Robertson DH, Cox KA, Gaskell SJ, Evershed RP, Beynon RJ. Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. Biochem J 1996;316:265–72.
[19] Mudge JM, Armstrong SD, Mclaren K, Beynon RJ, Hurst JL, Nicholson C, et al. Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. Genome Biol 2008;9:R91.
[20] Cheetham SA, Smith AL, Armstrong SD, Beynon RJ, Hurst JL. Limited variation in the major urinary proteins of laboratory mice. Physiol Behav 2009;96:253–61.
[21] Novikov SN, Churakov GA, Filimonenko AA, Ermakova II, Fedorova EM, Burkot IA. The pattern of major urinary proteins (MUPS) expression during postnatal ontogenesis of a laboratory mouse depends on genotype and sex. Ontogenez 2009;40:261–9.

[22] Armstrong SD, Robertson DH, Cheetham SA, Hurst JL, Beynon RJ. Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. Biochem J 2005;391:343–50.
[23] Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, Mclean L, et al. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. BMC Biol 2010;8:75.
[24] Mucignat-Caretta C, Redaelli M, Orsetti A, Perriat-Sanguinet M, Zagotto G, Ganem G. Urinary volatile molecules vary in males of the 2 European subspecies of the house mouse and their hybrids. Chem Senses 2010;35:647–54.
[25] Stopková R, Stopka P, Janotová K, Jedelský PL. Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). J Chem Ecol 2007;33:861–9.
[26] Grigsby CC, Rizki MM, Tamburino LA, Pitsch RL, Shiyanov PA, Cool DR. Metabolite differentiation and discovery lab (MeDDL): a new tool for biomarker discovery and mass spectral visualization. Anal Chem 2010;82:4386–95.
[27] Richmond BJ, Optican LM, Podell M, Spitzer H. Temporal encoding of two-dimensional patterns by single units in primate inferior temporal cortex. I. Response characteristics. J Neurophysiol 1987;57:132–46.
[28] Tashiro T, Mori K. Pheromone synthesis, CXCVII. Synthesis of the enantiomers of 2-*sec*-butyl-4,5-dihydrothiazole and (1*R*,5*S*,7*R*)-3,4-dehydro-*exo*-brevicomin, pheromone components of the male mouse, *Mus musculus*. Eur J Org Chem 1999;1999:2167–73.
[29] Tashiro T, Osada K, Mori K. Syntheses of 2-isopropyl-4,5-dihydrothiazole and 6-hydroxy-6-methyl-3-heptanone, pheromone components of the male mouse, *Mus musculus*. Biosci Biotechnol Biochem 2008;72:2398–402.
[30] Novotny M, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A. Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science 1986;231:722–5.
[31] Jemiolo B, Xie TM, Andreolini F, Baker AEM, Novotny M. The t complex of the mouse: chemical characterization by urinary volatile profiles. J Chem Ecol 1991;17:353–67.
[32] Jemiolo B, Gubernick DJ, Yoder MC, Novotny M. Chemical characterization of urinary volatile compounds of *Peromyscus californicus*, a monogamous biparental rodent. J Chem Ecol 1994;20:2489–500.
[33] Schwende FJ, Wiesler D, Jorgenson JW, Carmack M, Novotny M. Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. J Chem Ecol 1986;12:277–96.
[34] Pertinhez TA, Ferrari E, Casali E, Patel JA, Spisni A, Smith LJ. The binding cavity of mouse major urinary protein is optimised for a variety of ligand binding modes. Biochem Biophys Res Commun 2009;390:1266–71.
[35] Beauchamp GK, Yamazaki K. Chemical signalling in mice. Biochem Soc Trans 2003;31:147–51.
[36] Leinders-Zufall T, Brennan P, Widmayer P, Prashanth Chandramani S, Maul-Pavicic A, Jäger M, et al. MHC class I peptides as chemosensory signals in the vomeronasal organ. Science 2004;306:1033–7.
[37] Lazar J, Rasmussen LE, Greenwood DR, Bang IS, Prestwich GD. Elephant albumin: a multipurpose pheromone shuttle. Chem Biol 2004;11:1093–100.
[38] Dubois BW, Cherian SF, Evers AS. Volatile anesthetics compete for common binding sites on bovine serum albumin: a 19F-NMR study. Proc Natl Acad Sci U S A 1993;90:6478–82.
[39] Romeo JT. New SPME guidelines. J Chem Ecol 2009;35:1383.
[40] Osada K, Tashiro T, Mori K, Izumi H. The identification of attractive volatiles in aged male mouse urine. Chem Senses 2008;33:815–23.
[41] Garratt M, Stockley P, Armstrong SD, Beynon RJ, Hurst JL. The scent of senescence: sexual signalling and female preference in house mice. J Evol Biol 2011;24:2398–409.
[42] Novotny M, Harvey S, Jemiolo B. Chemistry of male dominance in the house mouse, *Mus domesticus*. Experientia 1990;46:109–13.
[43] Janotova K, Stopka P. The level of major urinary proteins is socially regulated in wild *Mus musculus musculus*. J Chem Ecol 2011;37:647–56.
[44] Harvey S, Jemiolo B, Novotny M. Pattern of volatile compounds in dominant and subordinate male mouse urine. J Chem Ecol 1989;14:2061–72.
[45] Singer AG, Tsuchiya H, Wellington JL, Beauchamp GK, Yamazaki K. Chemistry of odortypes in mice: fractionation and bioassay. J Chem Ecol 1993;19:569–79.
[46] Singer AG, Beauchamp GK, Yamazaki K. Volatile signals of the major histocompatibility complex in male mouse urine. Proc Natl Acad Sci U S A 1997;94:2210–4.
[47] Kwak J, Opiekun MC, Matsumura K, Preti G, Yamazaki K, Beauchamp GK. Major histocompatibility complex-regulated odortypes: peptide-free urinary volatile signals. Physiol Behav 2009;96:184–8.
[48] Hurst JL, Payne CE, Nevison CM, Marie AD, Humphries RE, Robertson DH, et al. Individual recognition in mice mediated by major urinary proteins. Nature 2001;414:631–4.
[49] Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, et al. Identification of protein pheromones that promote aggressive behaviour. Nature 2007;450:899–902.
[50] Sharrow SD, Vaughn JL, Zídek L, Novotny MV, Stone MJ. Pheromone binding by polymorphic mouse major urinary proteins. Protein Sci 2002;11:2247–56.
[51] Pes D, Pelosi P. Odorant-binding proteins of the mouse. Comp Biochem Physiol B Biochem Mol Biol 1995;112:471–9.
[52] Ramm SA, Cheetham SA, Hurst JL. Encoding choosiness: female attraction requires prior physical contact with individual male scents in mice. Proc Biol Sci 2008;275:1727–35.
[53] Beauchamp GK, Wysocki CJ, Wellington JL. Extinction of response to urine odor as a consequence of vomeronasal organ removal in male guinea pigs. Behav Neurosci 1985;99:950–5.
[54] Beauchamp GK, Martin IG, Wellington JL, Wysocki CJ. The accessory olfactory system: role in maintenance of chemoinvestigatory behavior. In: Müller-Schwarze D, Silverstein RM, editors. Chemical signals in vertebrates 3. New York: Plenum Press; 1983. p. 73–86.